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Short Communication

Immunohistochemical Detection of *Bcl*-2 in AIDS-Associated and Classical Kaposi's Sarcoma

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Kaposi's Sarcoma (KS) is an angioproliferative disease that is characterized by proliferation of spindle-shaped cells predominantly of vascular endothelial cell origin, neoangiogenesis, inflammatory cell infiltration, and edema. Although the lesions of classical KS and AIDS-associated KS (AIDS-KS) share common histological features, AIDS-KS occurs at a markedly higher frequency with a more aggressive clinical course. Immunohistochemical analyses of 26 evolutionarily staged AIDS-KS lesions derived from HIV-infected patients demonstrate significant cytoplasmic levels of Bcl-2, a protooncogene known to prolong cellular viability and to antagonize apoptosis. Bcl-2 expression increases as the pathological stage of KS advances. Immunohistochemical analyses of classical KS lesions demonstrate prevalent expression of Bcl-2 as well, indicating that upregulation of Bcl-2 may be important in the pathogenesis of both classical and AIDS-associated KS. Coexpression of Bcl-2 and factor VIII-related antigen in spindle-shaped cells present within KS lesions suggests that Bcl-2 is upregulated within the vascular endothelial spindle-shaped cells of KS. The consequences of upregulated Bcl-2 expression within KS lesions may be prolonged spindle cell viability which, when coupled with dysregulated cellular proliferation due in part to synergistic activities of inflammatory and angiogenic cytokines and HIV-1 Tat protein, may result in the maintenance, growth, and progression of KS. (Am J Pathol 1996, 148:1055–1063)

Kaposi's Sarcoma (KS) is an angioproliferative disease that is one of the most common neoplasias associated with AIDS (AIDS-KS).[1–3] In its classical form KS is rare, indolent, and found mainly in elderly men of Mediterranean origin; in contrast, AIDS-KS is frequent and aggressive, and occurs mostly in homosexual and bisexual men. Although genetic factors, sexually transmitted infectious agents, and immunosuppression may contribute to the pathogenesis of KS in general, cooperative effects of immunostimulation/dysregulation and HIV-1 infection appear to be of paramount importance in the development, maintenance and progression of AIDS-KS as compared with the classical form.[4,5] HIV-1 infection and/or other antigenic stimulation result in increased levels of inflammatory cytokines including tumor necrosis factor (TNF), interleukin (IL)-1, interferon-γ(IFN-γ), and IL-6 in serum and cultured leukocytes from HIV-1-infected individuals.[4–9] These same cytokines are increased in KS tissues,[9–10] and

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they stimulate the proliferation of spindle cells derived from KS lesions of AIDS patients and induce KS cell phenotypic and functional changes in normal vascular cells, the potential progenitors of KS spindle cells.[6,8–10] These cytokines also activate HIV-1 gene expression and replication, resulting in the concomitant production of the virally encoded regulatory protein Tat. After its release from infected cells, Tat promotes the proliferation of KS cells in a paracrine fashion and stimulates its own expression within HIV-1-infected cells via transactivation of HIV-1 long terminal repeat-directed gene expression.[5,6–8,10,11] Extracellular Tat can synergize with inflammatory cytokines and angiogenic factors such as basic fibroblast growth factor to promote angiogenesis and to act as a growth factor for KS spindle cells and cytokine-activated endothelial cells,[5,6,8,10–16] explaining, at least in part, the higher frequency and aggressiveness of AIDS-KS as compared with classical KS.

The proliferation of these spindle-shaped or "tumor" cells of KS is a hallmark of both AIDS-associated and classical KS progression; however, the factors affecting their sustained cellular viability and proliferation are only partially known. Tumor cells can evade normal mechanisms controlling cellular growth and survival by several mechanisms that include autocrine stimulation by tumor-derived cytokines and overexpression of specific cellular or viral oncogenes that may function as antagonists of apoptosis.[18–23] The protooncogene *Bcl-2*, which antagonizes apoptosis, promotes the prolonged survival of quiescent, noncycling cells and, unlike other oncogenes, fails to stimulate cellular proliferation.[24,25] *Bcl-2* overexpression renders cells less sensitive to radiation and cytotoxic drugs and inhibits apoptosis induced by dysregulated c-*myc* expression.[26,27] Transgenic mice that overproduce *Bcl-2* demonstrate prolonged B cell viability associated with high grade neoplasia.[26] The extended cell survival due to *Bcl-2* may lead to subsequent acquisition of genetic changes resulting ultimately in tumor progression.[26] *Bcl-2* appears to function, in part, by antagonizing apoptosis.[28,29] *Bcl-2* increases cell survival, without necessarily stimulating cellular proliferation[19]; therefore, coexpression of *Bcl-2* with factors that stimulate cellular proliferation may induce neoplastic transformation.

The primary objective of this study is to determine whether *Bcl-2* is expressed in the spindle-shaped endothelial cells of KS, suggesting its putative role in the pathogenesis of this disease. Data presented within this manuscript demonstrate, through immunohistochemical analyses, that spindle cells and mononuclear cell infiltrates derived from advancing AIDS-KS and classical KS lesions express significant levels of *Bcl-2*. *Bcl-2* overexpression and coexpression with factor VIII-related antigen (FVIII-RA) within a subpopulation of proliferating spindle-shaped cells within KS lesions is impressive, as normal vascular endothelial cells, the probable vascular progenitors of KS cells, fail to express significant levels of *Bcl-2*.[30] Increased expression of *Bcl-2* within KS lesions may prolong spindle cell viability. The increased viability, coupled with cytokine-induced cellular proliferation, likely contributes to the pathogenesis and progression of both AIDS-associated and classical KS.

Materials and Methods

Staging of KS Lesions

Both classical KS and AIDS-KS are characterized by similar histologies, including the presence of newly forming blood vessels, inflammatory cell infiltrates, edema, and proliferating spindle-shaped cells representing a heterogeneous cellular population dominated by vascular endothelial cells and few admixed dendritic and monocytic cells. The various evolutionary stages of 26 AIDS-KS and six classical KS lesions were determined by hemotoxylin and eosin staining. The detection of typical histological features facilitated staging of each of the KS lesions. In the patch or macular phases, the histological changes were confined mainly to the reticular dermis and especially to the areas around adnexal structures. Small primitive blood vessels dissecting collagen, few scattered extravasated red blood cells, rare plasma, and other mononuclear inflammatory cells were seen. In the intermediate or plaque stage, the degree of vascular proliferation involving the whole thickness of the dermis increased with processive lesional evolution. Within the deeper part of the dermis, spindle cell proliferation was seen with early beginnings of slit-like blood vessel formation. Hemosiderin pigments and a few scattered eosinophilic or hyaline globules were evident. Superficial layers of the dermis revealed dissecting blood vessels and a dense lymphocytic infiltrate. In the most advanced (nodular or tumor phase), the entire lesion presented as long spindle-shaped cells within a proliferating and intersecting fascicle-like region containing slit-like blood vessels, admixed erythrocytes, hemosiderin pigments, and eosinophilic bodies. Rare mitoses and anaplasias were seen involving the nuclei of the spindle-shaped cells.

Immunohistochemistry

Two immunohistochemical staining methods were used in this study, one for paraffin-embedded tissues and the other for fresh-frozen biopsies. Formalin-fixed, paraffin-embedded blocks containing 3 mm punch biopsies derived from AIDS-KS or benign ulceration and granulation tissues were processed and stained as previously described[31] with the following variations. Deparaffinization was performed by submerging sections in three changes of xylene (10 minutes each), followed by a series of graded ethanol (from 100 to 85%) and then distilled water. To block endogenous peroxidase activity, the sections were placed in 200 ml of 3% hydrogen peroxide for 10 minutes, followed by three washes in phosphate-buffered saline (PBS). Incubation with the primary antibody (mouse monoclonal anti-human *Bcl-2* oncoprotein antibody; DAKO-*Bcl-2*, clone 124, Glostrup, Denmark; diluted 1:10 with PBS) was done for 1 hour at room temperature and followed by streptavidin-biotin complex detection, as previously described.[32] The peroxidase reaction was initiated by the addition of 0.1% (w/v) 3,3′-diaminobenzidine solution for ≤10 minutes at room temperature. The positive control for Bcl-2 immunoreactivity represented formalin-fixed, paraffin-embedded sections from markedly reactive lymph node tissue. Immunoreactivity was graded as +1 through +4, respectively, when <10, between 10 and 25, between 25 and 50, and >50% of cells displayed staining with anti-*Bcl-2* antibody.

Frozen sections from AIDS-KS and classical KS lesions were fixed in cold acetone and singly or doubly stained by alkaline phosphatase-anti-alkaline phosphatase (APAAP) alone or combined with the peroxidase-anti-peroxidase (PAP) method by using mouse monoclonal antibody specific for human *Bcl-2* (DAKO, diluted 1:50) alone or in combination with a rabbit polyclonal antibody for FVIII-RA (DAKO; diluted 1:500) as described previously.[13,16] APAAP and PAP methods were then used to detect monoclonal and polyclonal antibodies, respectively. For single staining, the slides were incubated with monoclonal Bcl-2 antibody for 30 minutes at room temperature. After washing with Tris-buffered saline solution (TBS), the slides were incubated (20 minutes, room temperature) with rabbit antimouse immunoglobulin (Ig)G (DAKO; diluted 1:25). The slides were washed again in TBS and the APAAP (mouse) complex (1:25) was applied for an additional 20 minutes at room temperature. After washing, the second and third steps were repeated to amplify the reactions. The reaction was developed with the Fast Red substrate system (DAKO) and the slides were counterstained with Mayer's hematoxylin solution (Sigma Chemical Co., St. Louis, MO). The percentage of positive cells in duplicate samples for each experiment and in more than five fields per slide was determined microscopically. Immunoreactivity was graded as +1 through +4, respectively, when <10, between 10 and 25, between 25 and 50, and >50% of cells displayed staining with anti-Bcl-2 antibody. For double-staining (APAAP/PAP), the monoclonal antibody against *Bcl-2* was combined with polyclonal antibody against FVIII-RA. The universal DAKO double-stain kit system 40 was used according to manufacture's protocol.

Results

Expression of Bcl-*2 in AIDS-KS and Classical KS Lesions*

Formalin-fixed, paraffin-embedded tissues from 26 cases of AIDS-KS were classified into various evolutionary stages according to criteria described elsewhere,[33] and were tested for *Bcl-2* expression by immunohistochemistry with an anti-human *Bcl-2* monoclonal antibody. Seven ulcerative lesions from uninfected individuals were used as controls. Anti-*Bcl-2* staining revealed a cytoplasmic reactivity in 21 out of the 26 AIDS-KS cases tested. The intensity of immunoreactivity (% of positive cells) varied with the stage of the lesion (Table 1; Figure 1, A and B). Of the 8 cases examined in the macular stage, 4 revealed a *Bcl-2* reactivity of +1 (Table 1; Figure 1, left), whereas the remaining cases were nonreactive. Of the 13 cases examined in the plaque or intermediate phase, 12 cases were positive for *Bcl-2* expression (Table 1 and Figure 1, middle panel). Among them, five lesions, six lesions, and one lesion showed a +1, +2, and +3 reactivity, respectively (Table 1, Figure 1). Generally, the regions of the lesions containing abundant spindle-shaped cells displayed the most immunoreactivity with the *Bcl-2* antibody; however, proliferating blood vessels with prominent endothelial cells were reactive as well. Mature blood vessels and postcapillary venules in the upper part of the dermis (papillary dermis) showed no immunoreactivity. All five cases in the nodular or tumor stage displayed reactivity with anti-*Bcl-2* antibody (Table 1; Figure 1, right), with four cases reflecting an intensity of +2 and one case having an intensity of +3. Comparison of anti-*Bcl-2* immunoreactivity in all cases representing lesions evolving from macule to plaque and nodule stages demonstrated a relative increase

Table 1. *Clinicopathological Data and Anti-*Bcl-*2 Immunohistochemical Reactivity on 26 AIDS-KS Cases*

Case no.	Age	Sex	Race	Location	Clinical history	Histopathology	Anti-*Bcl-2* immunoreactivity
1	40	M	C	Leg	Purple nodule	Macule	+
2	35	M	C	Chest	Nodule	Macule	+
3	33	M	C	Chest	Violaceous plaque	Plaque	+++
4	34	M	C	Leg	Violaceous plaque	Macule	–
5	31	M	C	Shoulder	Purple papule	Plaque	++
6	35	M	C	Leg	Purple papule	Plaque	++
7	56	M	C	Leg	Violaceous plaque	Plaque	+
8	28	M	C	Leg	Violaceous plaque	Plaque	+
9	28	M	B	Shoulder	Erythematous lesions	Plaque	++
10	31	M	C	Leg	Violaceous plaque	Nodule	++
11	28	M	C	Leg	Purple papule	Plaque	–
12	34	M	C	Leg	Purple nodule	Nodule	++
13	34	M	C	Toe	Purple nodule	Nodule	++
14	33	M	C	Thigh	Purple papule	Macule	+
15	25	M	B	Arm	Purple nodule	Nodule	++
16	35	M	C	Leg	Violaceous plaque	Macule	–
17	35	F	B	Wrist	Violaceous plaque	Macule	–
18	24	F	B	Arm	Violaceous plaque	Plaque	+
19	37	M	B	Shoulder	Violaceous plaque	Macule	–
20	31	M	H	Foot	Purple plaque	Plaque	+
21	31	M	H	Arm	Purple plaque	Plaque	+
22	30	M	B	Abdomen	Violaceous plaque	Plaque	++
23	43	M	C	Groin	Violaceous plaque	Plaque	++
24	33	M	B	Leg	Violaceous plaque	Macule	+
25	39	M	C	Leg	Violaceous plaque	Plaque	++
26	25	M	C	Leg	Purple nodule	Nodule	+++

Letters M, F, C, B, and H represent male, female, caucasian, black, and Hispanic, respectively. Symbols +, ++, +++ and ++++ indicate <10%, between 10 and 25%, between 25 and 50%, and >50% cells displaying positive anti-*Bcl-2* immunoreactivity, respectively. Anti-*Bcl-2* staining was performed on formalin-fixed AIDS-KS lesions as described in Materials and Methods.

in *Bcl-2* expression as lesions progress toward the nodular or "tumor" stage (Figure 1B). Two of the seven control cases of benign ulcers showed positive (+1) immunoreactivity with anti-*Bcl-2* antibody (Table 2).

To determine whether classical KS lesions similarly express significant levels of Bcl-2, frozen sections from six classical KS patients were analyzed for *Bcl-2* immunoreactivity by the APAAP method. All of the six cases of classical KS examined revealed a strong anti-*Bcl-2* immunoreactivity (Figure 2, right), as compared with uninvolved skin from the same patient (Figure 2, left) or control staining (Figure 2, middle). The percentages of *Bcl-2*-positive cells per high power microscopic field ranged from 33–37% (+3 immunoreactivity) in three of six cases to 63–70% (+4 immunoreactivity) in three of six cases. As observed in AIDS-KS, higher *Bcl-2* positivity correlated with more advanced lesions, containing more spindle-shaped cells. Frozen sections from AIDS-KS lesions and from uninvolved normal skin that were simultaneously stained with the classical KS lesions revealed positive and negative anti-*Bcl-2* immunoreactivity, respectively (Figure 3A), confirming the results obtained on formalin-fixed tissues (Table 1, Figure 1).

Coexpression of Bcl-*2 and Factor VIII in Spindle Cells of AIDS-KS Lesions*

KS lesions represent a heterogeneous population of cells, some of which acquire a spindle-shaped morphology and proliferate as the stage of the lesion progresses. Previous studies suggested that these spindle cells are composed of a heterogenous population of cells dominated by activated endothelial cells, while other spindle-shaped cells are of macrophage origin.[16,34] To determine whether endothelial spindle cells co-stain for *Bcl-2*, AIDS-KS lesions were doubly stained with anti-*Bcl-2* and anti-FVIII-RA antibodies combined. These immunohistochemical analyses demonstrated that a large proportion of *Bcl-2*-positive spindle cells coexpressed FVIII-RA, indicating that upregulated *Bcl-2* expression is occurring within KS cells of vascular endothelial cell origin (Figure 3, A and B). In addition, *Bcl-2* and FVIII-RA coexpression was also found in endothelial cells of vessels (Figure 3B). Other *Bcl-2*-positive cells were most likely of mononuclear origin, representing lymphocytes and macrophages infiltrating the lesions.

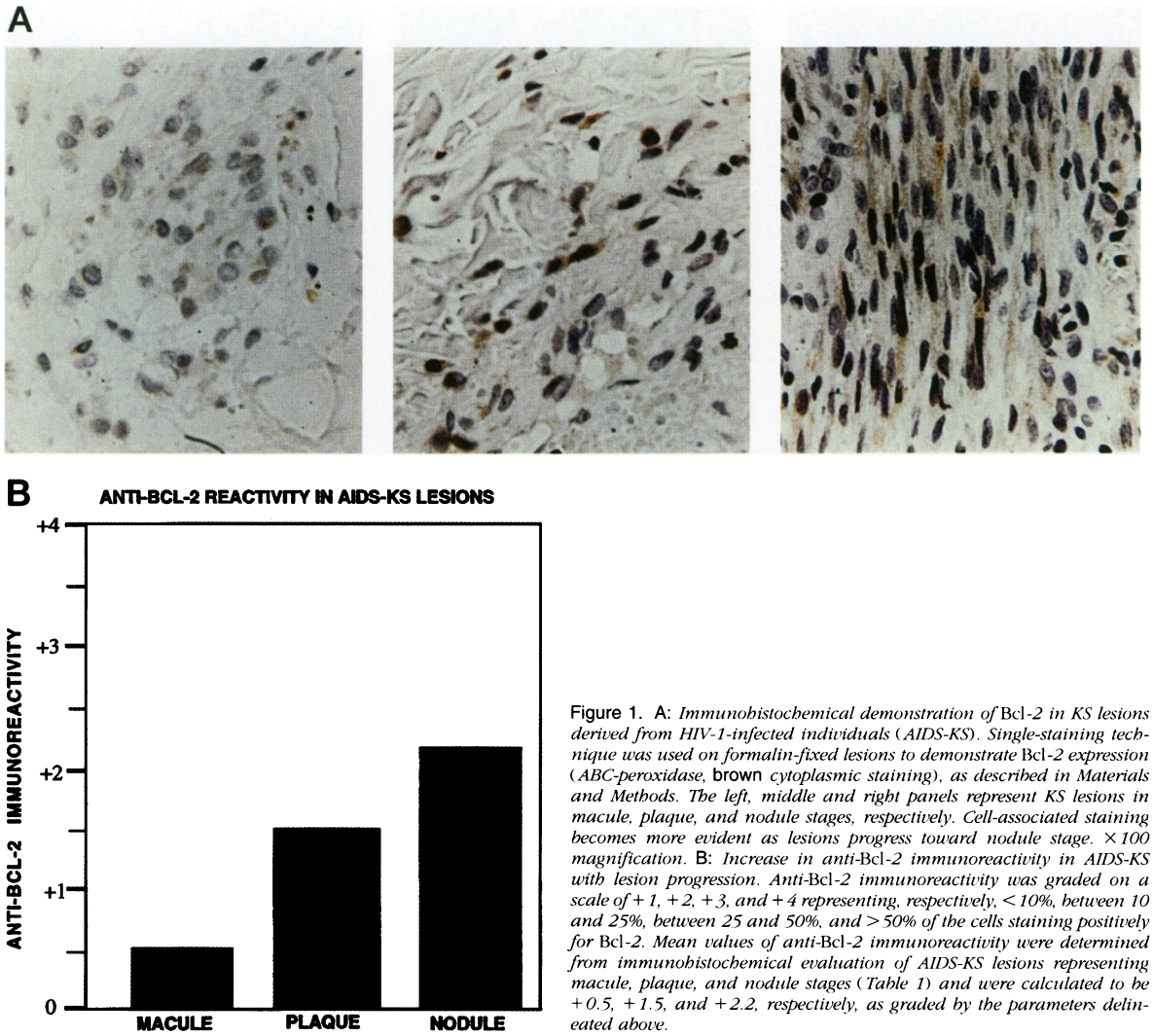

Figure 1. A: *Immunohistochemical demonstration of* Bcl-*2 in KS lesions derived from HIV-1-infected individuals (AIDS-KS). Single-staining technique was used on formalin-fixed lesions to demonstrate* Bcl-*2 expression (ABC-peroxidase,* brown *cytoplasmic staining), as described in Materials and Methods. The left, middle and right panels represent KS lesions in macule, plaque, and nodule stages, respectively. Cell-associated staining becomes more evident as lesions progress toward nodule stage. ×100 magnification.* B: *Increase in anti-*Bcl-*2 immunoreactivity in AIDS-KS with lesion progression. Anti-*Bcl-*2 immunoreactivity was graded on a scale of +1, +2, +3, and +4 representing, respectively, <10%, between 10 and 25%, between 25 and 50%, and >50% of the cells staining positively for* Bcl-*2. Mean values of anti-*Bcl-*2 immunoreactivity were determined from immunohistochemical evaluation of AIDS-KS lesions representing macule, plaque, and nodule stages (Table 1) and were calculated to be +0.5, +1.5, and +2.2, respectively, as graded by the parameters delineated above.*

Table 2. *Clinicopathological Data and Anti-*Bcl-*2 Immunohistochemical Reactivity in Seven Cases of Benign Ulcers*

Case no.	Age	Sex	Race	Location	Clinical history	Histopathology	Anti-*Bcl-2* immunoreactivity
1	62	F	C	Leg	Non-healing ulcer	Ulcer with granulation tissue	–
2	65	M	C	Foot	Non-healing ulcer	Ulcer with granulation tissue	–
3	58	F	C	Leg	Non-healing ulcer	Ulcer with granulation tissue	+
4	44	M	C	Thigh	Ulcerated nodule	Ulcerated acrochordon	–
5	37	F	C	Leg	Non-healing ulcer	Ulcer with granulation tissue	–
6	14	M	C	Leg	Non-healing ulcer	Ulcer with granulation tissue	–
7	15	M	C	Buttock	Non-healing ulcer	Ulcer with granulation tissue	+

Letters M, F and C represent male, female and caucasian, respectively. Symbols +, ++, +++ and ++++ indicate <10%, between 10 and 25%, between 25 and 50%, and >50% cells displaying positive anti-*Bcl*-2 immunoreactivity, respectively. Anti-Bcl-2 staining was performed on formalin-fixed tissues as described in Materials and Methods.

Discussion

Immunohistochemical analyses demonstrate that spindle cells derived from advancing AIDS-KS and classical KS lesions express significant cytoplasmic levels of the cellular oncogene, *Bcl*-2, a known antagonist of apoptosis. *Bcl*-2 expression increases with lesion progression, and the highest levels are observed in the nodular stage of the disease. Further, coexpression of *Bcl*-2 and FVIII-RA in spindle cells and vessels indicates that induction of *Bcl*-2 within vascular endothelial cells may play an impor-

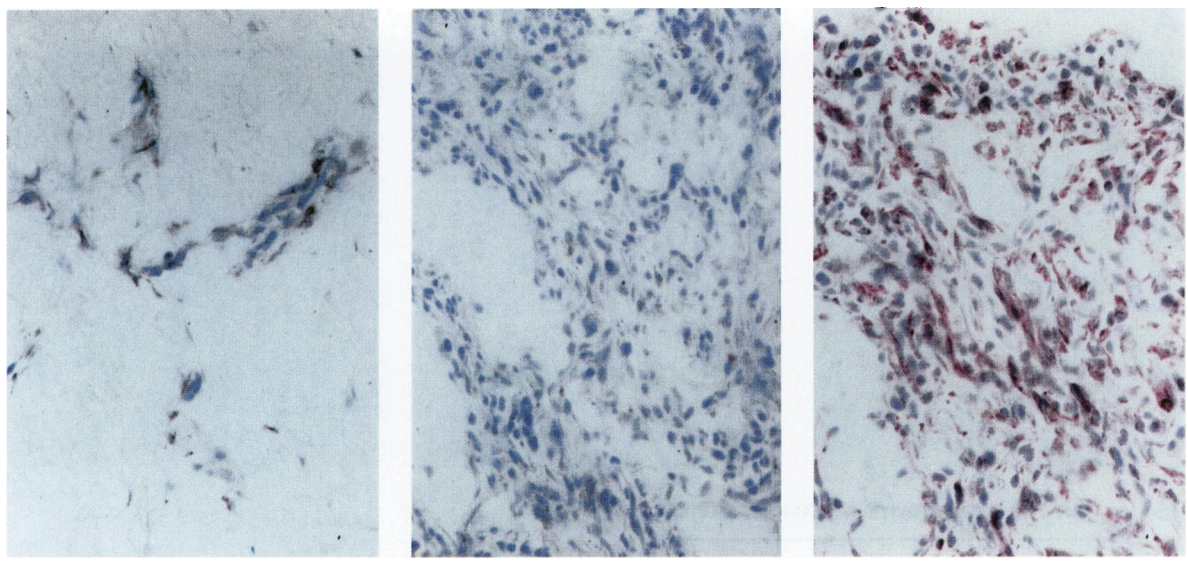

Figure 2. *Immunohistochemical demonstration of* Bcl-*2 in classical KS lesions. Single-staining technique was used on frozen sections from classical KS or uninvolved skin to demonstrate* Bcl-*2 expression (APAAP,* red *cytoplasmic staining). The left, middle and right panels represent immunohistochemical analyses of uninvolved skin with anti-*Bcl-*2 antibody, classical KS tissue with phosphate-buffered saline (control), and classical KS tissue with anti-*Bcl-*2 antibody, respectively. ×40 magnification.*

tant role in the pathogenesis of KS whereby induction of Bcl-2 expression within KS endothelial cells and stimulation of cell growth by inflammatory and angiogenic cytokines or Tat may provide coordinate cellular survival and proliferative signals to affect the maintenance and progression of KS.

Histologically, AIDS-associated and classical KS are indistinguishable; yet, KS within HIV-1-infected individuals has a comparatively aggressive clinical course.[1–3] The pathogenic mechanisms to explain this more aggressive form of KS are not completely understood, although cooperative effects of immunostimulation/dysregulation and HIV-1 infection appear to have significant influence on the development, maintenance, and progression of AIDS-KS.[4,5,16] In early stages, AIDS-KS appears to be strictly a cytokine-mediated disease where angiogenic and inflammatory cytokines and HIV-1 Tat cooperate in its induction and progression.[4,5,16] The cooperative effects of viral and cellular factors within AIDS-KS result in unregulated cellular proliferation and angiogenesis. Additionally, recent data demonstrate that late stage KS can transform into a true tumor[35]; therefore, our finding of increased *Bcl-2* expression that correlates with advancing KS lesions, particularly evident within the nodular-tumor stage of the disease, suggests that *Bcl-2* expression may serve as a molecular marker for tumor progression in KS.

Immunohistochemical analyses of AIDS-KS lesions obtained as fresh punch biopsies from HIV-1-infected patients have demonstrated the presence of HIV-1 Tat protein.[16] The ability of Tat to rescue cultured epithelial, neuronal, and lymphoid cells from apoptosis induced by serum deprivation appears to correlate with increased levels of *Bcl-2* within these cells.[36] Thus, upregulated expression of *Bcl-2* within advancing AIDS-KS lesions shown in the present study may occur through a cytokine-regulated pathway that is augmented by the presence of Tat. These several studies suggest, cumulatively, that Tat may indirectly influence the induction of *Bcl-2* expression in AIDS-KS.

Bcl-2 expression in KS lesions may also be influenced by viral gene products other than HIV-1 Tat. It has recently been demonstrated that a newly identified human herpesvirus (KSHV) has been associated with both classical and AIDS-associated KS.[37] Although its role in the pathogenesis of KS is as yet unknown, this virus may also play a contributory role in regulating *Bcl-2* expression and potentiating cell survival as suggested by previous studies of herpesviral-associated *Bcl-2* expression in which upregulation of *Bcl-2* and consequent blocking of apoptosis by viral gene products (with or without cooperative effects of cytokines) have been described. In particular, a recent study demonstrates putative, cooperative effects of IFN-γ and herpes simplex virus-2 in the induction of *Bcl-2* and in the associated survival of infected neurons.[38] Additionally, the latent membrane protein-1 of another member of the herpesvirus family, the Epstein-Barr virus (EBV), has been demonstrated to upregulate *Bcl-2* expression and block apoptosis in infected B-cell lines.[39] Similarly,

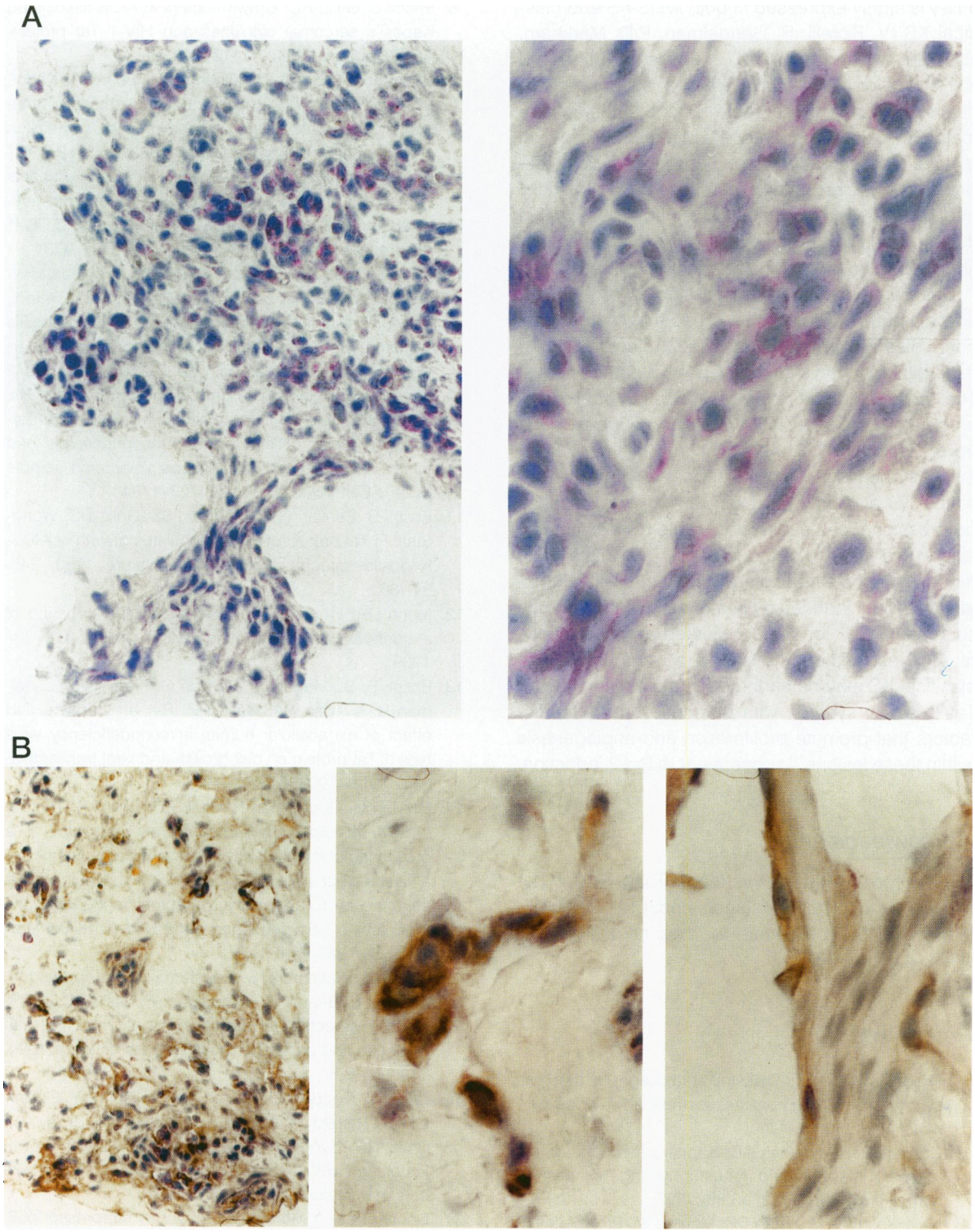

Figure 3. **A** *and* **B:** *Coexpression of* Bcl-*2 and FVIII-RA in AIDS-KS. Double-staining immunohistochemical technique was used on frozen sections from AIDS-KS lesions to demonstrate expression of* Bcl-*2 (APAAP,* **red** *cytoplasmic staining) and FVIII-RA (PAP,* **brown** *cytoplasmic staining).* **A:** *the left (×40 magnification) and right (×400 magnification) panels represent double-staining of AIDS-KS lesions using anti-*Bcl-*2 monoclonal antibody and phosphate-buffered saline (control).* **B:** *the left (×40 magnification), middle (×400 magnification) and right (×400 magnification) panels represent double-staining of AIDS-KS lesions using anti-*Bcl-*2 monoclonal antibody and anti-FVIII-RA polyclonal antibody. The middle and right panels represent two different fields (×400) of the same tissue. Uninvolved skin tissues were used as controls (data not shown).*

IFN-γ is highly expressed in both AIDS-KS and classical KS (V. Fiorelli, R. Gendelman, P.D. Markham, R.C. Gallow, and B. Ensoli, unpublished data) and KSHV and/or EBV[38] are detected by polymerase chain reaction in these same lesions. Thus, inflammatory cytokines and direct or indirect effects of viral factors may similarly affect upregulated *Bcl-2* expression in KS, resulting in increased cellular viability and growth responsiveness of spindle cells within these lesions to paracrine and autocrine inflammatory cytokines and angiogenic factors or Tat.

Direct immunohistochemical studies of AIDS-KS and classical KS lesions, such as those presented here, disclose novel clues toward elucidating the molecular mechanisms involved in KS pathogenesis. The presence of overexpressed *Bcl-2* in both AIDS-associated and classical KS lesions suggests that upregulation of *Bcl-2* expression may be important in the pathogenesis of both classical and AIDS-associated KS and may represent a marker of disease progression. *Bcl-2* expression within KS lesions may extend the viability of the KS cells and make them more susceptible to proliferative effects of inflammatory cytokines induced by immunostimulatory agents such as KSHV[37] or HIV-1.[8] Marked aggressiveness of AIDS-associated KS lesions as compared with classical KS may be attributed to the ability of HIV-1 Tat to act in synergy with paracrine and autocrine factors that promote proliferation and angiogenesis within these lesions[16] subsequent to *Bcl-2* induction.

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